Fish Physiol Biochem (2018) 44:49–62
DOI 10.1007/s10695-017-0412-x

Effects of epinephrine exposure on contractile performance of compact and spongy myocardium from rainbow trout (*Oncorhynchus mykiss*) during hypoxia

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Received: 8 March 2017 / Accepted: 21 July 2017 / Published online: 9 August 2017

Abstract Hypoxia results in elevated circulating epinephrine for many fish species, and this is likely important for maintaining cardiac function. The aims of this study were to assess how hypoxia impacts contractile responses of ventricular compact and spongy myocardium from rainbow trout (*Oncorhynchus mykiss*) and to assess how and if epinephrine may protect myocardial performance from a depressive effect of hypoxia. Work output and maximum contraction rate of isolated preparations of spongy and compact ventricular myocardium from rainbow trout were measured. Tissues were exposed to the blood PO_2 that they experience in vivo during environmental normoxia and hypoxia and also to low (5 nM) and high (500 nM) levels of epinephrine in 100% air saturation (PO_2 20.2 kPa) and during hypoxia (PO_2 2 kPa, 10% air saturation). It was hypothesized that hypoxia would result in a decrease in work output and maximum contraction rate in both tissue types, but that epinephrine exposure would mitigate the effect. Hypoxia resulted in a decline in net work output of both tissue types, but a decline in maximum contraction rate of only compact myocardium. Epinephrine restored the maximum contraction rate of compact myocardium in hypoxia, appeared to slightly enhance work output of only compact myocardium in air saturation but surprisingly not during hypoxia, and restored net work of hypoxic spongy myocardium toward normoxic levels. These results indicate hypoxia has a similar depressive effect on both layers of ventricular myocardium, but that high epinephrine may be important for maintaining inotropy in spongy myocardium and chronotropy in compact myocardium during hypoxia.

Keywords Epinephrine · Rainbow trout · Myocardium · Contractile performance · Hypoxia

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Introduction

Environmental hypoxia causes a decline in the partial pressure of oxygen (PO_2) of blood in fish (Thomas et al. 1994; Gamperl et al. 1994a). Exposure to acute hypoxia can also elicit an endocrine stress response (Perry and Reid 1992). For example, rainbow trout (*Oncorhynchus mykiss*) exposed to a drop in water PO_2 (P_wO_2) to 4.6 kPa experience an increase in circulating epinephrine (from 1.9 to 300 nM) and norepinephrine (from 1 to 100 nM) (Perry and Reid 1992). Increases in circulating catecholamines are important in the primary stress response of fish and are known to affect physiological parameters related to oxygen delivery (Iwama et al. 1999; Tuurala et al. 1982).

Elevated circulating catecholamines result in an increase in cardiac contractility and cardiac output (Axelsson et al. 1990; Farrell et al. 1986; Gamperl et al. 1994b) mediated through the stimulation of adrenergic receptors (AR) on the myocardium and in the vasculature (Vornanen 1998; Zhang et al. 1998). Likewise, direct exposure to epinephrine increases the work

output of isolated rainbow trout hearts and isolated ventricular myocardium (Farrell et al. 1986; Shiels et al. 1998), including in mammals (Layland et al. 1997). Through the use of pharmacological agents, the roles of the different AR have been characterized for their effects on circulation and the heart (Petersen et al. 2013). The AR subtypes currently identified in fish are the α-AR, β_1-AR, β_2-AR, and β_3-AR (Nickerson et al. 2003). Activation of different receptor subtypes can elicit different and sometimes opposing effects on the myocardium and vasculature (Imbrogno et al. 2015), with the overall effects of catecholamine exposure on the inotropy and chronotropy of the myocardium likely reflecting the distribution of these AR subtypes on the cell surfaces.

While the effects of catecholamines on cardiac function in hypoxia have been investigated to some extent (see above), the specific impacts on the different tissue layers of the heart are not known. The ventricle of rainbow trout is divided into two morphologically distinct myocardial tissues: the outer compact layer of myocardium perfused with arterial blood supplied by a coronary artery and the inner spongy layer only receiving oxygen through the returning venous blood (Farrell and Jones 1992). This anatomical configuration creates a situation where the spongy myocardium is constantly exposed to poorly oxygenated venous blood relative to the compact layer perfused by the coronary supply. In hypoxic conditions, the PO_2 of venous blood (P_vO_2) drops even further (Thomas et al. 1994); for example, in well-oxygenated water, P_wO_2 is near 20 kPa, arterial PO_2 (P_aO_2) in rainbow trout is 13 kPa, and P_vO_2 is much lower at about 5 kPa (Gamperl et al. 1994a). When exposed to acute environmental hypoxia of 12 kPa (about 60% air saturation), both the P_aO_2 and P_vO_2 decline. However, while P_aO_2 perfusing the compact myocardium (4.7–6 kPa) still remains higher than P_vO_2 (2–3.3 kPa) that perfuses the spongy layer (Gamperl et al. 1994a), the decline in P_aO_2 is much greater in magnitude than the decline in P_vO_2. Hence, the compact layer will experience a much greater decline in PO_2 during hypoxia than the spongy layer, and this may have implications not only for overall cardiac performance, but also for the relative performance of each layer and how they contribute to a functional heart. The spongy layer is associated with volume pumping (high flow rates but at relatively low pressure), while the compact layer, when present, is associated with pressure pumping (high pressure but with relatively low volumes) (Agnisola and Tota 1994). Hence, hypoxia and resulting changes in the relative contributions of the two tissue layers could alter the function of the heart.

In addition to differences in PO_2 experienced by the compact and spongy layers, they may express different β-AR subtypes and densities and thus respond differently to adrenergic stimulation. For example, the spongy layer of Coho salmon (*Oncorhynchus kisutch*) has a 14% higher surface density of β-AR than the compact myocardium (Gamperl et al. 1998). It is currently unknown if this difference in surface receptor densities has an effect on the adrenergic response of the two tissue layers. Differences in sensitivity to epinephrine between the tissue layers could allow for maintained cardiac function despite differences in oxygen availability during hypoxia between the tissue layers. The work output of ventricular muscle from Atlantic cod (*Gadus morhua*), in the absence of epinephrine, decreases rapidly following exposures to lower PO_2 (Syme et al. 2013). However, high levels of epinephrine may allow cardiac output to be maintained during hypoxia and help prevent cardiac collapse (Hanson et al. 2006), as observed in isolated compact ventricular strips from rainbow trout during anoxia (Gesser et al. 1982).

In addition to effects of catecholamines on force production by the myocardium, there appears to be an impact on heart rate, where catecholamines tend to promote tachycardia. As evidence, depression of the heart rate in rainbow trout occurs with the injection of propranolol, a β_1-AR and β_2-AR antagonist (Petersen et al. 2013). Additionally, during exercise, circulating epinephrine levels increase concurrently with heart rate (Butler et al. 1986), although this response has not been observed consistently (Gamperl et al. 1994b). Adrenergic stimulation is known to increase the sarcolemmal calcium current density in fish myocardial cells (Vornanen 1998) and to affect the phosphorylation state of phospholamban which in turn affects pumping of calcium into the sarcoplasmic reticulum by SERCA (Shiels and Galli 2014), both of which could alter twitch kinetics and the maximum sustainable contraction rate of myocardium.

The aims of this study were to measure the contractile performance of spongy and compact myocardium under physiologically relevant PO_2 during normoxia and hypoxia and additionally to measure the contractile performance of these tissues during exposure to low and high

levels of epinephrine in both air saturation and under hypoxic conditions. It was hypothesized that (1) the larger drop in PO_2 experienced by the compact myocardium during hypoxia would result in a greater loss in work output relative to spongy; (2) exposure to lower PO_2 would result in a reduction in maximum sustainable heart rate in both ventricular tissues; (3) exposure to high levels of epinephrine would result in an increase in the work output of compact and spongy myocardium, potentially restoring the work output of myocardium during hypoxia; and (4) high epinephrine would result in an increase in the maximum sustainable contraction rate for both myocardial tissue layers.

Methods

Animal handling and tissue isolation

All aspects of animal handling were approved by the animal care committee at the University of Calgary following CCAC guidelines. Experiments were conducted on tissue isolates of 23 freshwater-acclimated rainbow trout of mixed sex, obtained from the Sam Livingston fish hatchery in Calgary, Alberta, Canada. All fish were exposed to a 16-:8-h light/dark photoperiod and held in 100% air-saturated water at 14 °C. The holding tank was 1000 l. Trout were fed ~1% body weight of commercial trout pellets daily (Martin Mills Inc. Classic sinking fish feed for salmonids). Fish were allowed to acclimate to these conditions for 3 months prior to experimentation.

Fish were removed from the tank and euthanized with a sharp percussion to the cranium followed by pithing the brain and spinal cord. Immediately following euthanasia, the hearts were extracted and placed in chilled saline: in mM, NaCl 132, KCl 2.6, $CaCl_2$ 2.7, $MgSO_4$ 1.0, NaH_2PO_4 1.0, glucose 10, and HEPES buffer 10, pH = 7.8 (adapted from Altringham and Johnston 1990). From each heart, strips of compact and spongy myocardium were removed from the ventricle. Preparations were dissected out attempting to maintain similar dimensions between experiments. To reduce the effects of diffusion limitations in the tissues, the radius of all preparations were maintained near or below 0.5 mm. For preparations of spongy myocardium, whole trabeculae were selected based on a columnar shape with minimal apparent branching of the muscle fibers. Since muscle fiber alignment is not superficially apparent for compact myocardium, preparations were selected based on the apparent alignment of fibers as observed during spontaneous contractions.

Following the dissections, segments of 6-0 silk suture were tied on either end of the preparations and preparations were attached via these sutures to the arm of a servo motor (model 350, Cambridge Technology Inc., Bedford, MA) and a force transducer (model 400a, Aurora Scientific, Aurora, ON, Canada). Mounted preparations were bathed in saline held at 14 °C throughout the experiment.

Dissections and experiments were first completed for one tissue type (~90 min), while the remaining ventricular tissue was dissected to near its final dimensions and left pinned under light tension in chilled saline, the saline was replaced every 20 min, electrical stimulus was regularly applied every 10 min, and air was bubbled into the saline to maintain a high PO_2. After completion of experiments on the first tissue type (spongy or compact, in random order), experiments were then conducted on the second tissue type.

Measuring work output from isolated myocardium

Data were collected, and experimental parameters were controlled with software custom-written using LabView (ver 6.1, National Instruments, Austin, TX) and a 12-bit analog/digital converter card (PCI MIO 16E-4, National Instruments, Austin, TX). Muscle preparations were stimulated to elicit contractions with a stimulator (Isostim A320, WPI, FL, USA) that allowed control of voltage and stimulus pulse duration. Pulse duration was set to 1 ms, and the voltage was set approximately 50% above that needed for maximum force production. The stimulator was connected to two platinum plates placed on either side of the submerged muscle preparation.

The work-loop method was used to measure work output of the muscle preparations, with repeated cycles of lengthening and shortening (strain) simulating contractions of a beating heart (e.g., Syme and Josephson 1995; Layland et al. 1997; Harwood et al. 1998; Shiels et al. 1998; Syme et al. 2013). Shortening of the muscle simulates strain during systole, while lengthening emulates ventricular filling during diastole. The frequency of the strain cycles and electrical stimulation allowed simulation of different heart rates. The servomotor applied strain in a sinusoidal pattern with an amplitude of 10% peak to peak of the muscle's resting length in both the spongy and compact preparations.

The resting length of each preparation was optimized to maximize work output before experiments began. To do so, preparations were subjected to a series of 5 cycles of strain at a cycling frequency of 50 beats per minute (bpm), with the net work output from the final strain cycle used for comparison. Optimal length was found by increasing the preparation length in increments of 0.1 mm between series of work measurements until work output no longer increased following an increase in preparation length. This optimum length was then used for all subsequent measures from the muscle.

Experiment 1: contractile performance during exposure to in vivo PO_2

To measure the effects of PO_2 on contractile performance, preparations of both spongy and compact myocardium were exposed, in a random order, to the blood PO_2 that they would experience in vivo during normoxia and hypoxia. The normoxic PO_2 was 65% air saturation (13 kPa) for compact preparations and 25% air saturation (5 kPa) for spongy. The hypoxic PO_2 was 25% air saturation (5 kPa) for compact and 10% air saturation (2 kPa) for spongy. These values were selected based on in vivo P_aO_2 and P_vO_2 in rainbow trout exposed to hypoxic P_wO_2 of 60% air saturation (12 kPa) (Gamperl et al. 1994a). For a rainbow trout, P_wO_2 of 60% air saturation is well above the PO_2 where cardiac failure occurs and above the P_wO_2 where hypoxic bradycardia occurs (Gamperl et al. 1994a). Measurements were made on tissues from 11 fish.

PO_2 in the saline bathing the muscle was controlled by bubbling with mixtures of nitrogen and atmospheric air using a gas mixing pump (Wosthoff M200, Bochum, Germany). PO_2 was monitored in the saline by a fiber optic meter (Presens Fibox 3 LCD, Espoo, Finland). Once the desired PO_2 of the saline was achieved, the preparations were exposed to three sets of 30 consecutive strain and contraction cycles at 50 bpm to assess and attain stability of the preparation before measurements commenced. Once stable, three more sets of 30 strain cycles were completed, and the net, lengthening, and shortening work output of the final strain cycles in each set were recorded and averaged for the analysis. After making recordings at a given PO_2, the preparations were then returned to 100% air saturation to avoid any cross-over effects of prolonged hypoxia exposure before being exposed to the remaining PO_2 to be tested (either hypoxic or normoxic, depending which was tested first) and the measurement procedure was repeated.

Experiment 2: contractile performance with epinephrine

The effects of high (500 nM) and low (5 nM) epinephrine exposures during high (100% air saturation) and low (10% air saturation) PO_2 exposures on work and maximum contraction rate were measured in both spongy and compact myocardium. The same PO_2 was used for both tissue types (i.e., 100 and 10% air saturation), rather than the in vivo PO_2 as described for experiment 1, to avoid the potentially confounding effects on interpretation of having different PO_2 exposures between tissue types. Measurements were made on tissues from 12 fish. Stocks of epinephrine (Sigma-E4375) were prepared in saline and frozen at −10 °C for a maximum of 1 week before use; stocks were thawed just prior to use during experiments and kept on ice wrapped in aluminum foil to minimize exposure to light.

With the PO_2 of the saline initially set at 100% air saturation (20.2 kPa), an aliquot of epinephrine stock was added to the saline bath to bring the total saline concentration to 5 nM, which is similar to blood concentrations in unstressed rainbow trout (Perry and Reid 1992). The saline was then allowed to circulate for 1 min before measurements of work output and maximum contraction rate were made. To reduce the effects of photodegradation on epinephrine concentration, all measurements of contractile performance at a given PO_2 were completed within 10 min of epinephrine addition and the experimental chamber was covered with a sheet of aluminum foil. Muscle preparations were subjected to three sets of 30 consecutive strain cycles and contraction to assess work output, in the same manner as described in experiment 1. Then, the maximum contraction rate was measured, analogous to the maximum heart rate before the myocardium would experience arrhythmia. For this, the preparations were held at their optimal resting length and subject to a series of stimulations while measuring isometric force. A set of 30 contractions at 50 bpm was first applied to attain a stable response from the preparation. Then, ten contractions were recorded at each of 60, 70, etc. up to 140 bpm, with no interruption between each set of contractions. At slower contraction rates, the force was consistent and stable between successive contractions (Fig. 1). However, at high stimulation rates, the force began to

oscillate between successive contractions, being high then low then high, etc. (Fig. 1). The maximum contraction rate was taken as the highest rate the preparation could be stimulated where the developed isometric force remained stable between successive contractions (i.e., force of the last two contractions in the set of 10 remained within 10% of each other); stimulation at higher frequencies resulted in force oscillating by more than 10% between successive contractions, and the muscle was considered arrhythmic.

Following completion of these measurements in 100% air saturation with 5 nM epinephrine, the saline in the chamber was replaced to avoid accumulation of epinephrine. The PO_2 was then lowered to 10% air saturation (2 kPa), and fresh epinephrine stock was added to re-establish a concentration of 5 nM, and the measurements of work and maximum contraction rate were repeated. The saline was then replaced and PO_2 returned to 100% air saturation, but now with a high concentration of epinephrine (500 nM), and the same series of measurements were repeated. The high concentration of epinephrine is similar to that experienced in trout stressed by hypoxia (Perry and Reid 1992). Finally, the saline was again replaced and measurements were repeated at 10% air saturation and 500 nM epinephrine. Measurements in low epinephrine always preceded those in high to avoid the possibility of high epinephrine exposure carrying over to measurements in low.

Trout and preparation size

The trout used in experiment 1 had an average standard length of 19.1 ± 0.63 cm (mean ± SE), with an average mass of 105 ± 9 g and a ventricular mass of 124.0 ± 8.2 mg. In experiment 2, the trout had a standard length of 24.8 ± 0.75 cm with an average mass of 189 ± 11 g and a ventricular mass of 204.1 ± 10.5 mg. For experiment 1, the mass of compact preparations was 2.2 ± 0.37 mg and for the spongy myocardium 0.63 ± 0.078 mg. The optimal length of compact preparations was 4.17 ± 0.39 mm and in the spongy myocardium 2.53 ± 0.23 mm. For experiment 2, compact preparations had a mass of 2.7 ± 0.40 mg and spongy myocardium was 1.9 ± 0.42 mg. The optimal length for the compact preparations was 4.77 ± 0.32 mm and for the spongy preparations was 3.57 ± 0.41 mm.

Analysis

Following completion of an experiment, preparations were removed from the apparatus and viewed under a microscope; tissue that was clearly non-viable or distal to the suture ties was dissected away. The preparation was then blotted on a filter paper to remove surface moisture and weighed (Mettler-Toledo MT5, Highston NJ, USA). Work was then expressed relative to muscle mass (J/kg) to facilitate comparison between tissue layers.

For all analyses of work output, the average work value and coefficient of variance were calculated from the three measures of work made under each experimental condition. If the coefficient of variance was greater than 15%, the triplicate work value most different from the other two was removed for analysis. For experiment 1, this was done for 8 out of 76 total measurements; for the other 68, all three values of work were included in the average. In experiment 2, this was done for 19 out of 89 total observations. There were two instances where the CV remained higher than 15% even after the exclusion of a value; these work values were excluded from analysis.

All analyses were conducted using R software, and mixed effect models were assessed using the lme4 package (Bates et al. 2005; R-core team 2015). For all models, a random intercept term was included for individual preparations, accounting for the non-independence of the repeated measures. Diagnostic residual plots were used to visually assess normality of residuals: histogram, QQ norm, and Pearson residual vs. Fitted plots. *P* value estimates were obtained from the package lmerTest using Satterthwaite estimation of degrees of freedom (Hrong-Tai Fai and Cornelius 1996; Kuznetsova et al. 2013). Where significant differences were found, Tukey's correction factors were applied to post hoc contrasts.

For experiment, 1 the effects of normoxic and hypoxic PO_2 on net, shortening, and lengthening work (J/kg) were analyzed with the main effects: PO_2 (normoxia or hypoxia), tissue (compact or spongy), and the interaction term between the two main effects included. Net and shortening work were log transformed to improve the fit of residuals to a normal distribution.

For experiment 2, log-transformed work and untransformed maximum contraction rate were compared between low and high epinephrine exposures at 100% air saturation for both tissue types, with the fixed effects of tissue type, epinephrine concentration, and an epinephrine-tissue-type interaction. The same fixed

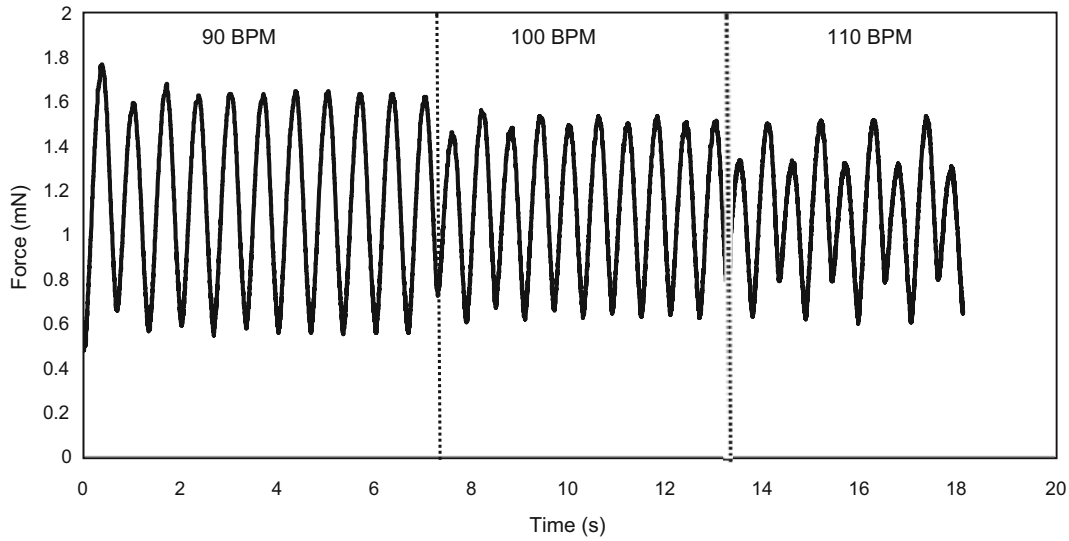

Fig. 1 Isometric twitch force from spongy myocardium during a series of stimulations at increasing frequency; only data at 90, 100, and 110 bpm are shown. The twitches at 90 and 100 bpm were considered stable, as force oscillates less than 10% between successive contractions, while at 110 bpm, the muscle is considered arrhythmic as force oscillates more than 10% between successive contractions. Maximum contraction rate for this preparation is defined as 100 bpm, the highest frequency that contractions were stable

effect structure was used to compare the proportion of net, shortening and lengthening work output in hypoxia relative to that produced in air saturation (i.e., work output at 10% air saturation/work output at 100% air saturation) during exposure to low and high epinephrine levels. Maximum contraction rates were compared between high and low epinephrine at 10% air saturation for both tissue types, with the same fixed effect as in 100% air saturation. Maximum contraction rates were compared between tissue types at 100 and 10% air saturations with low epinephrine. The fixed effects for this model were PO_2, tissue-type, and the PO_2-tissue-type interaction. Finally, maximum contraction rates were compared between 100% air saturation in low epinephrine and 10% air saturation in high epinephrine. The fixed effects for this mixed effect model were treatment (100% air saturation low epinephrine, 10% air saturation high epinephrine), tissue type, and the treatment-tissue-type interaction.

Results

Work output with physiologically relevant PO_2

At the PO_2s experienced in vivo for fish in environmental normoxia and hypoxia, hypoxia resulted in a significant decline in net work ($P = 0.002$) and shortening work ($P = 0.005$), but did not affect lengthening work in either tissue type ($P = 0.17$) (Fig. 2). Post hoc contrasts showed that PO_2 had a significant effect on net work in both compact ($P = 0.001$) and spongy myocardium ($P = 0.008$), but on shortening work of only the compact myocardium ($P < 0.001$), not the spongy ($P = 0.20$). There was no difference between tissue types nor an interaction between PO_2 and tissue type for net work (tissue type $P = 0.35$, PO_2-tissue interaction $P = 0.18$) or for shortening work (tissue type $P = 0.24$, PO_2-tissue-type interaction $P = 0.23$). However, lengthening work was significantly higher in spongy vs. compact myocardium at the in vivo PO_2 during both normoxia and hypoxia ($P = 0.035$). There was no interaction of PO_2 and tissue type for lengthening work ($P = 0.55$).

Effects of epinephrine on work

In 100% air saturation, there was no significant difference between the work produced by compact and spongy myocardium when compared at similar concentrations of epinephrine ($P = 0.71$) (Fig. 3). High epinephrine led to a significantly higher net and shortening work output ($P = 0.003$, $P = 0.029$, respectively), but only for the compact myocardium (net $P = 0.003$,

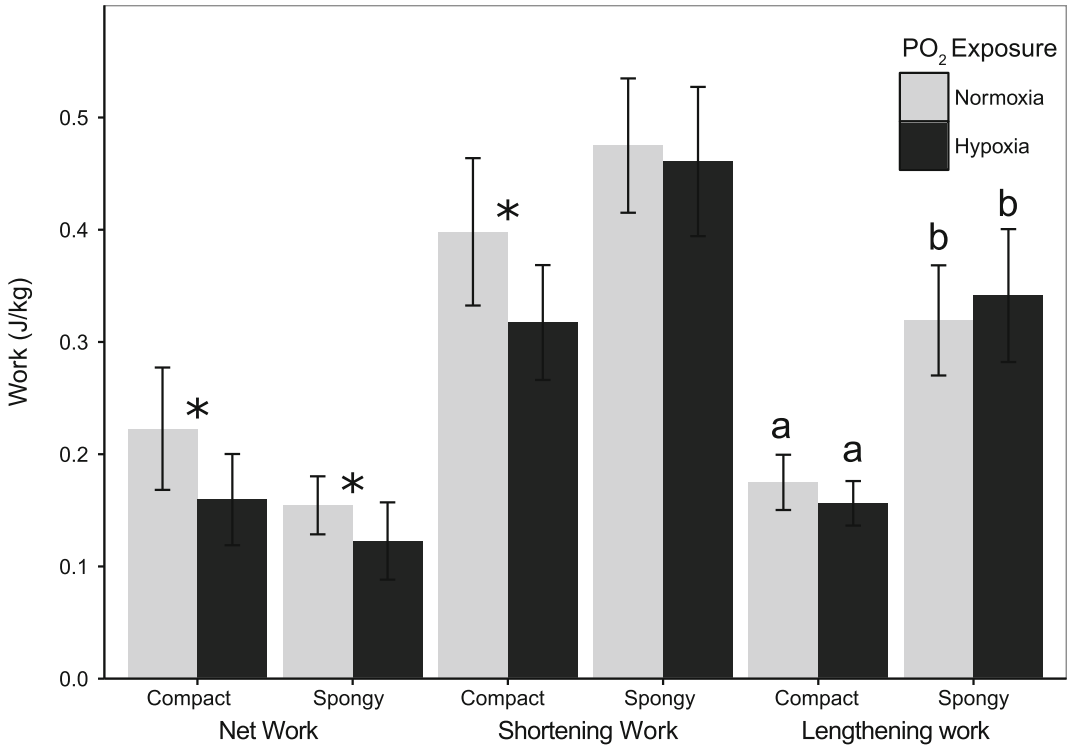

Fig. 2 Mass-specific net, shortening, and lengthening work output of spongy and compact myocardium at the PO_2s experienced by the respective tissue in vivo when fish are in environmental normoxia and hypoxia (compact normoxia 65% air saturation, compact hypoxia 25% air saturation, spongy normoxia 25% air saturation, spongy hypoxia 10% air saturation). *Asterisks* indicate significant difference between normoxia vs. hypoxia ($P < 0.05$). Lengthening work was significantly higher in spongy vs. compact in both normoxia and hypoxia ($P = 0.035$); *similar letters* denote no significant difference, while *different letters* denote significant difference. Data are means ± SEM. $N = 11$

shortening $P = 0.045$), not the spongy myocardium (net $P = 0.14$, shortening $P = 0.24$). The effect of epinephrine on lengthening work was marginally not significant ($P = 0.052$) (Fig. 3). The interaction between tissue type and epinephrine was not significant (net $P = 0.27$, shortening $P = 0.56$, lengthening $P = 0.23$).

In 10% air saturation, the proportion of net and shortening work output maintained relative to what was produced in 100% air saturation was greater in high versus low epinephrine (net $P = 0.020$, shortening $P = 0.010$) (Fig. 4); however, the effect was significant only in spongy myocardium (net $P = 0.025$, shortening $P = 0.029$), not compact (net $P = 0.34$, shortening $P = 0.14$). Despite there being a significant effect of epinephrine only in spongy myocardium, there was not a statistically significant difference between tissue types in the proportion of net and shortening work maintained in high vs. low epinephrine (net $P = 0.12$, shortening $P = 0.43$), with no significant tissue type-epinephrine exposure interaction (net $P = 0.22$, shortening $P = 0.43$). The proportion of lengthening work maintained in 10% air saturation was not significantly different between tissue types ($P = 0.76$), epinephrine concentration ($P = 0.63$), or the interaction ($P = 0.61$).

Effects of epinephrine on maximum contraction rate

When compared with 100% air saturation, exposure to 10% air saturation resulted in a significant reduction in the maximum contraction rate in low epinephrine ($P = 0.006$) (Fig. 5), but the effect was only significant in compact myocardium ($P < 0.001$), not spongy myocardium ($P = 0.14$). There was not a significant PO_2-tissue-type interaction ($P = 0.38$). At 100% air saturation, high epinephrine did not affect the maximum

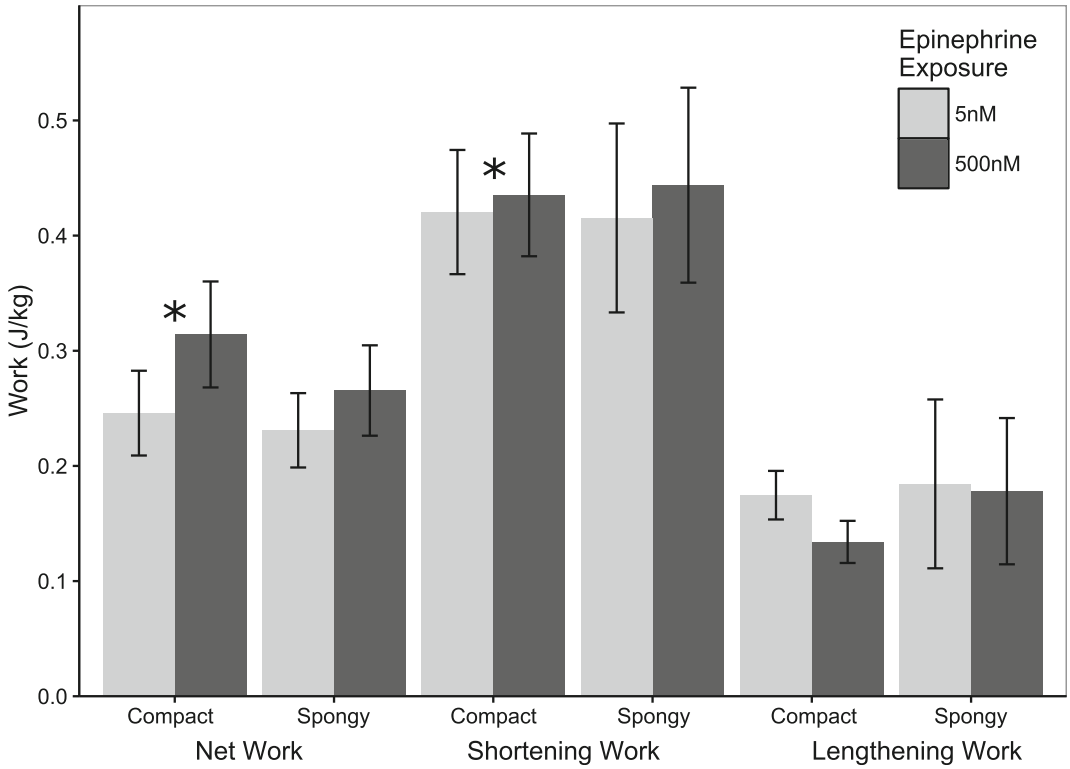

Fig. 3 Mass-specific net, shortening, and lengthening work output from compact and spongy myocardium at 100% air saturation with low (5 nM) and high (500 nM) levels of epinephrine. *Asterisks* indicate significant difference between low and high epinephrine levels ($P < 0.05$). Data are means ± SEM. $N = 12$

contraction rate, for either spongy or compact myocardium ($P = 0.13$) (Fig. 5). However, in 10% air saturation, high epinephrine resulted in a significantly higher maximum contraction rate ($P = 0.023$), but again, the effect was significant only in compact myocardium ($P = 0.006$), not spongy ($P = 0.66$). In both 100 and 10% air saturations, as well as low and high epinephrine, spongy myocardium had a significantly higher maximum contraction rate than compact (100% air saturation $P < 0.001$, 10% air saturation $P < 0.001$). Finally, maximum contraction rates in 100% air saturation and low epinephrine (i.e., as might be expected in normoxia) were not significantly different from those in 10% air saturation and high epinephrine (i.e., as might be expected in hypoxia) ($P = 0.70$) (Fig. 5).

Discussion

Work output during hypoxia

Both compact and spongy myocardium exhibited a decrease in net work when exposed to the PO_2 that they would experience during environmental hypoxia, relative to performance in normoxia (Fig. 2). However, the basis of the response differed somewhat between tissue types. The loss of net work in compact myocardium was mediated only through a decrease in shortening work, as lengthening work was not affected by hypoxia (Fig. 2). This pattern of change in work during hypoxia matches what has previously been observed in ventricular spongy myocardium of Atlantic cod at 10 °C during hypoxia (Syme et al. 2013). In contrast, while spongy myocardium also exhibited a loss in net work in hypoxia, this was not associated with a significant change in either shortening or lengthening work (Fig. 2); it appears that the non-significant trend of decreased shortening work and increased lengthening work in hypoxia was sufficient to result in a significant reduction in net work in spongy myocardium.

Both tissue types exhibited a similar relative decrease in net work following exposure to hypoxia (Fig. 2). However, the absolute magnitude of

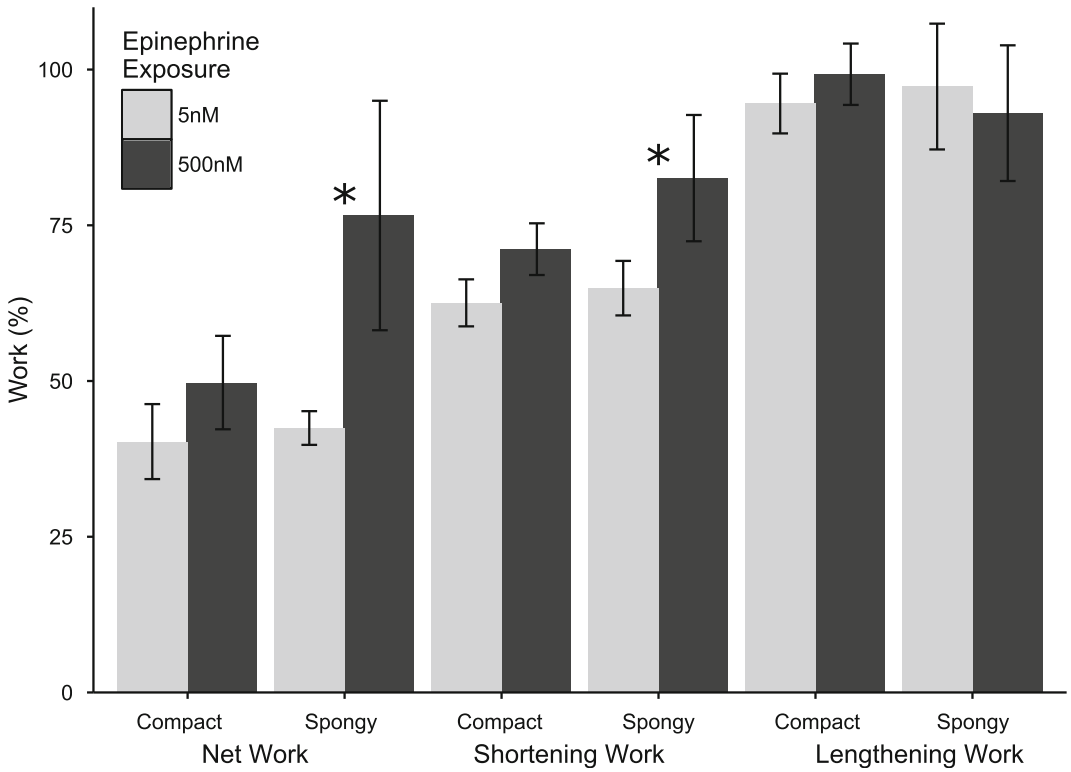

Fig. 4 Net, shortening, and lengthening work output at 10% air saturation (hypoxia), expressed as a proportion (%) of work produced at 100% air saturation, from compact and spongy myocardium with low (5 nM) and high (500 nM) levels of epinephrine. *Asterisks* indicate significant difference between low and high epinephrine levels ($P < 0.05$). Data are means ± SEM. $N = 12$

the drop was larger in compact, and this is associated with a larger drop in PO_2 from normoxia to hypoxia in compact than in spongy myocardium. A larger drop in PO_2 might induce proportionately greater reliance on anaerobic metabolism in the compact myocardium, which in turn could be associated with a larger reduction in work output. In support of the notion that both tissue layers respond similarly to changes in PO_2, the compact and spongy myocardium exhibited a similar decline in net work when exposed to the same change in PO_2 (100 to 10% air saturation), although only when exposed to low levels of epinephrine (Fig. 4). Likewise, when exposed to the same decrease in PO_2, both tissue layers showed a similar drop in shortening work (with low epinephrine) (Fig. 4). Further, when exposed to hypoxia as experienced in vivo, where the compact experiences a much larger drop in PO_2 than spongy, only the compact myocardium showed a significant drop in shortening work (Fig. 2). Thus, despite differences between the tissue layers in the PO_2 that they routinely experience, they appear to exhibit similar sensitivities to hypoxia.

The clear decline in net work output during hypoxia appears to contrast with other measures of cardiac performance in vivo during hypoxia. It has been shown in several species, including Atlantic cod and rainbow trout, that cardiac output is largely maintained during hypoxic conditions above critical values (Fritsche and Nilsson 1989; Gamperl et al. 1994a). This indicates that additional factors are affecting cardiac performance during hypoxia in vivo, despite a decline in the capacity of the muscle to perform work. The increase in adrenergic tone associated with hypoxia is likely involved in this relationship (Perry and Reid 1994; Reid et al. 1998). However, the environmental P_wO_2 that would result in the hypoxic PO_2 used in the present study (i.e., the in vivo PO_2 used in experiment 1) would not be low enough to elicit a substantial increase in circulating epinephrine (Perry and Reid 1992). It is thus unlikely that the maintenance of cardiac output during this level

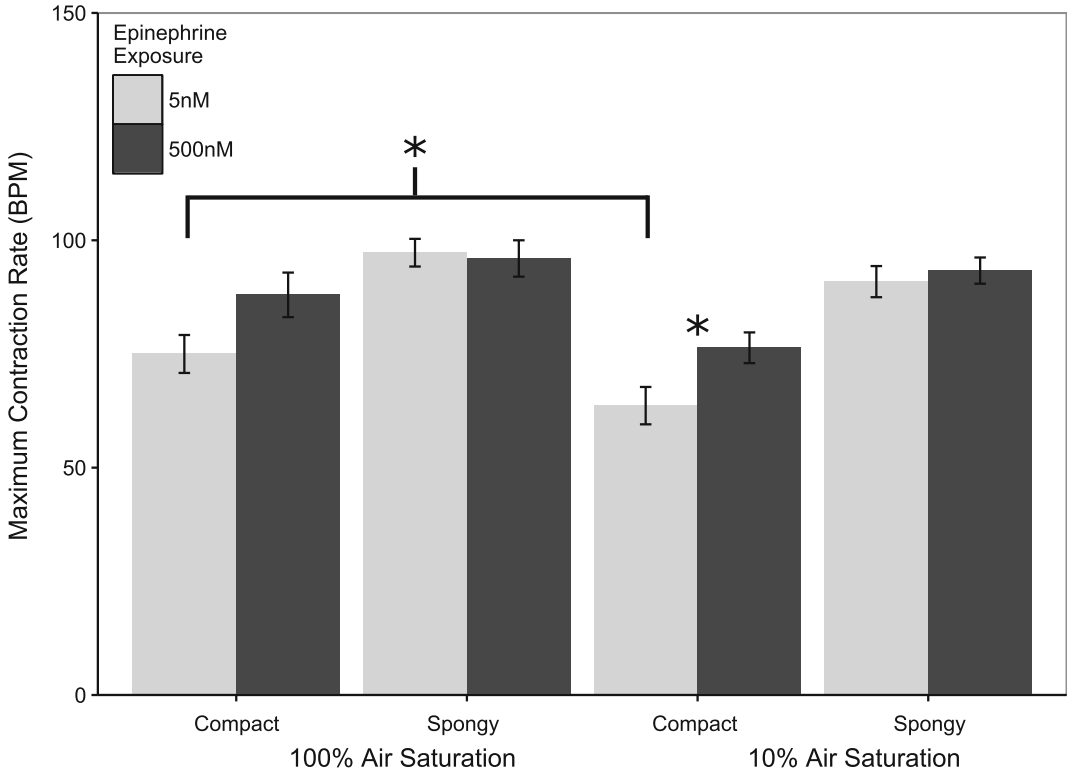

Fig. 5 Maximum contraction rate (bpm) of spongy and compact myocardium at 100 and 10% air saturations with low (5 nM) and high (500 nM) levels of epinephrine. *Asterisks* indicate significant difference between low and high epinephrine levels or between 100 and 10% air saturations (indicated with *bracket* for compact myocardium) ($P < 0.05$). Spongy had a significantly higher maximum contraction rate than compact under all comparable conditions ($P < 0.001$). Data are means ± SEM. $N = 12$

of hypoxia in vivo is due solely to a systemic adrenergic stress response. Other factors, including humoral, mechanical, and neural, are likely involved. Further, cardiac muscle in vivo is likely not normally functioning at its maximal capacity in normoxia, providing it with some scope to sustain the same level of performance during hypoxia.

Effects of epinephrine on work

In 100% air saturation, high epinephrine resulted in a small increase in shortening and net work from compact myocardium (Fig. 3), with no impact on lengthening work, as predicted. However, epinephrine did not increase inotropy in the spongy myocardium (Fig. 3). This is in contrast to results of Shiels et al. (1998), where work output of spongy myocardium of rainbow trout increased with high epinephrine exposure in well-oxygenated conditions. The discrepancy may be explained by the 20-fold lower concentration of adrenaline used in the present study (500 nM) vs. the study by Shiels et al. (10 μM). We chose 500 nM as a concentration similar to that reported in salmonids during hypoxic stress (Perry and Reid 1992). Regardless, even with 10 μM epinephrine, the increase in work from spongy myocardium was relatively small, about 25%, similar to the increase in work from compact myocardium with 500 nM epinephrine noted in the present study (Fig. 3). Thus, it appears that when oxygen levels are relatively high, there is the potential for a small increase in contractility of spongy and compact myocardium through adrenergic stimulation, but higher levels of epinephrine may be required to elicit this response in spongy myocardium.

The lack of an effect of epinephrine on spongy myocardium (in high oxygen), or perhaps a higher dose required to elicit a response, contrasts with expectations based on a higher β-AR surface receptor density in spongy vs. compact myocardium of adult, wild Coho salmon (Gamperl et al. 1998). However, we do not know if these tissue

differences extend to rainbow trout. It has also been noted that the hearts of Coho salmon have a 2.8× higher β-AR surface receptor density than those of hatchery-reared rainbow trout (Gamperl et al. 1998). Thus, the relatively lower β-AR densities for myocardium from the hatchery-reared trout, as used in the present study, may contribute to a different adrenergic response from what is expected based on Coho salmon. Further, β-AR surface receptor density may be variable across individual fish, contributing to a similar average increase in net work in both tissue types (compact average 15% and spongy average 16%), but leading to variability that precluded a statistically significant effect in spongy (Fig. 3). Also, there could be different receptor subtypes present on the tissues resulting in a different response (see discussion below). Overall, the small impact of high epinephrine on compact myocardial work, and the lack of an effect on spongy myocardium, suggests that these isolated tissues were working at or near their maximal capacity when oxygen levels are relatively high.

Unlike in 100% air saturation, with 10% air saturation, high epinephrine resulted in significantly more net work and shortening work from the spongy myocardium, but not compact (Fig. 4). Ten percent air saturation is much closer to the in vivo PO_2 for spongy myocardium at all P_wO_2. Thus, the adrenergic response at 10% air saturation for the spongy myocardium might be more representative of an in vivo response to elevated epinephrine and is consistent with the improved hypoxia tolerance of cardiac function in rainbow trout with high epinephrine (Hanson et al. 2006). This reversal in tissue-specific responses to epinephrine in 100 vs. 10% air saturation may indicate changes to adrenergic sensitivity in hypoxia. Increases in cyclic AMP production (a downstream effector in the signal transduction pathway of β_2-AR activation) and increases in receptor density during acute hypoxia (<60 min) have been observed in erythrocytes of rainbow trout (Reid et al. 1993). Similar mechanisms may operate in the myocardium during hypoxia, perhaps to differing degrees in spongy vs. compact myocardium.

The lack of an effect of epinephrine on work output of compact myocardium at 10% air saturation (Fig. 4), despite a small effect at 100% air saturation (Fig. 3), contrasts with observations of increased developed isometric twitch force of compact myocardium in high epinephrine during anoxia (Gesser et al. 1982). This discrepancy may again be the result of higher epinephrine concentrations being used in that study (5 μM) than in the present study (500 nM). The difference in response between 100 and 10% air saturations may also reflect differences in β-AR subtypes present on the compact myocardium. Both β2-AR and β3-AR have been identified in ventricular myocardium of rainbow trout (Motyka et al. 2016; Petersen et al. 2013). β_3-AR stimulation acts on myocardium to decrease inotropy (Imbrogno et al. 2015) and involves the oxygen-sensitive signal transduction molecule nitric oxide (Imbrogno et al. 2015) which could confer a PO_2 sensitivity to the β-AR response of the myocardium.

Effects of hypoxia and epinephrine on maximum contraction rate

The compact myocardium showed a decline in the maximum contraction rate between 100 and 10% air saturations when exposed to low levels of epinephrine (Fig. 5). A decrease in maximum contraction rate, as defined by an inability to sustain force consistently between contractions, may reflect changes to the cardiac action potential and calcium homeostasis. This could be caused by a reduction of the calcium current as a result of L-type Ca^{2+} channel inactivation or other disruptions in Ca^{2+} handling (Shiels et al. 2002) or alterations in membrane potential. There are rapidly O_2-sensitive L-type Ca^{2+} channels in rat myocardium (Scaringi et al. 2013), and ischemic ventricular arrhythmia in mammals is thought to be associated with a reduction of intracellular K^+ through depression of the Na/K pump (Janse and Wit 1989). If similar sensitivities exist in trout ventricular myocardium, this could potentially affect the ability of the heart to sustain high contraction rates.

In vivo, hypoxic bradycardia in fish is largely mediated by changes in vagal tone (Short et al. 1979; Farrell 2007; Mckenzie et al. 2009). Thus, while a hypoxia-induced slowing of heart rate is not necessarily mechanistically coupled to a reduction in the intrinsic maximum contraction rate of the myocardium (Fig. 5), they may be related. This is supported by observations in vagotomized Atlantic cod which still experience bradycardia during deep hypoxia and in European eels (*Anguilla anguilla*) where arrhythmias are observed following atropine treatment in hypoxia (Mckenzie et al. 2009; Iversen et al. 2010). As well, in mammals, ischemia caused by coronary blockage can result in arrhythmias

of the myocardium (Janse and Wit 1989). Hypoxic bradycardia may serve to preserve cardiac function by supressing heart rate to avoid arrhythmias that could result from the hypoxic decline in maximum sustainable contraction rate.

High epinephrine appeared to fully reverse the hypoxia-induced decline in maximum contraction rate of the compact myocardium, where the maximum contraction rate of compact myocardium in 10% air saturation with high epinephrine was not different from that in 100% air saturation with low epinephrine (Fig. 5 and see "Results" section). This suggests a protective effect of epinephrine. Petersen et al. (2013) observed that application of the β_3-AR-specific agonist BRL-37344 resulted in an increase in heart rate of adult rainbow trout. In mammals, β_3-AR stimulation results in a briefer action potential (Gauthier et al. 1996), which could enhance the maximum contraction rate of the myocardium. Thus, perhaps, adrenergic stimulation of β_3-AR on the trout compact myocardium helps restore maximal contraction rate in hypoxia.

Of note, spongy myocardium had a higher maximum contraction rate than the compact myocardium, and neither hypoxia nor exposure to high epinephrine at any PO_2 had a significant effect on maximum contraction rate of spongy myocardium (Fig. 5). The maximum contraction rate of the spongy myocardium thus appears very insensitive and stable. The difference in maximal rate between the two tissue layers is reduced with high epinephrine in both high and low oxygen, although it is not eliminated (Fig. 5). It is unclear why the spongy myocardium would possess a higher maximal contraction rate than compact, as the two layers must always contract in unison in a functional heart. It may be that the difference does not have a functional significance and simply reflects differences in the cardiac action potential or calcium handling between the tissue layers.

Future directions and conclusions

Further research is needed to clarify the mechanistic bases of the adrenergic responses. Additionally, the effect of norepinephrine exposure is required to more fully understand the role of hypoxic catecholamine release on cardiac function. This may be particularly important, as norepinephrine has a higher binding affinity to β_3-AR, and norepinephrine levels are also highly elevated during hypoxia (Perry and Reid 1992; Imbrogno et al. 2015).

The adrenergic response in compact myocardium appears to be PO_2 dependent and could potentially reflect a balance in β-AR subtype activity. The depression of maximum contraction rate in hypoxia and the increase in maximum contraction rate in response to epinephrine for compact myocardium suggest that elevated circulating catecholamines may be important to preserve the ability to raise the heart rate during hypoxia in trout. Additionally, high epinephrine during acute hypoxia may help preserve inotropy, particularly of the spongy myocardium. As the spongy myocardium makes up ~70% of the rainbow trout ventricle, an increased relative contribution of spongy myocardium during hypoxia, compared to compact, suggests a shift in the pumping mechanics of the heart in hypoxia toward high flow but lower pressure.

Acknowledgements We would like to thank the Natural Sciences and Engineering Research Council of Canada for funding. We would also like to thank Kimberly Stewart for technical assistance in conducting experiments.

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